Surface immobilization and bioactivity of TGF-β1 inhibitor peptides for bone implant applications

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KEYWORDS:
Biofunctionalization, Silanization, TGF-β inhibitor peptides, Titanium, Surface modification.

ABSTRACT
TGF-β1 is the most related cytokine with the production of fibrotic tissue. It plays an important role on the production of collagen by fibroblasts and other types of cells. The inhibition of this cytokine has demonstrated to reduce fibrosis in various types of tissue. Biofunctionalization of dental and orthopedic implants with biomolecules enables modification of the physical, chemical and biochemical properties of their surfaces to improve its biological and clinical performance. Our objective was to develop a reliable method to immobilize oligopeptides on Ti surfaces to obtain a surface with TGF-β1 inhibitory activity that will potentially minimize fibrotic encapsulation of implants during the process of osseointegration. We covalently immobilized TGF-β1 inhibitory P17-peptides on Ti surfaces and assessed by characterizing each step of the process that we successfully biofunctionalized the implant surfaces. High amounts of peptides were anchored and homogeneously distributed on the surfaces with mechanical and thermochemical stability after in vitro simulated challenges. Notably, the immobilized peptides retained their TGF-β1 inhibitory activity in vitro. Thus, these coatings represent a promising new approach for biofunctionalizing surfaces for inducing a fast and reliable osseointegration.
INTRODUCTION

Implant surgery provokes a series of reactions from the host organism such as inflammation, wound healing, foreign body reaction and fibrosis. Transforming Growth Factor Beta 1 (TGF-β1) has been proved to play an important role in the host reaction to the implant. Immediately after surgery, cells surrounding the implant increase the production of TGF-β1 entailing the production of collagen type I, which is the main component of the fibrous tissue. However, the beneficial or detrimental effects of TGF-β1 on the osseointegration of an implant are not yet clear. Endoosseous metallic implants are encapsulated by an undesirable fibrous tissue layer with thickness that depends on the virulence of the host reaction and micro-displacements between the implant and the bone bed. This formation of the fibrous tissue layer may lead to bad vascularization of peri-implant tissues and thus, low stability of the implant may occur.

Borrás Cuesta et. al. developed a peptide, P17, that inhibits the TGF-β1 signaling pathway and as a result of that reduces fibrosis in liver injury in rats. P17 (H-KRIWFIPRSSWYERA-OH) is a TGF-β1 inhibitor peptide that binds TGF-β1 and blocks TGF-β1 interaction with cell receptors. Therefore, cells reduce the production of collagen type I and fibrotic reactions.

Biofunctionalization of titanium surfaces opens new perspectives of development of titanium implants. Surface coatings made of biological molecules with known biological activities let to tailor the biochemical properties of implant surfaces having, therefore, a better control of biomaterials-tissue interactions. Biochemical modification of titanium surfaces using appropriate peptides can alter adhesion of specific cells on the surface, influence their differentiation rate, reduce healing times, and increase long-term stability of the implant. Biochemical modification of titanium surfaces can be achieved by a variety of different methods. These methods involve physical adsorption or chemical binding. Chemical binding is often
the chosen alternative due to the generation of an irreversible covalent bond between the surface and the immobilized molecule with high stability under the harsh in vivo environmental conditions. However, it is a more challenging process than physical adsorption as the immobilization method requires the formation of a specific, strong and stable bonds between the organic molecules and the inorganic substrate. Biochemical immobilization methods using organic linking molecules such as silanes \(^{21,22}\), phosphonates \(^{23,24}\), thiols \(^{24}\) and polyethylene glycol (PEG) \(^{25}\) have been studied to decorate surfaces with biological molecules. 3-aminopropyltriethoxy silane (APTES) has been widely used to tether bioactive peptides to a number of oxide surfaces \(^{26,27}\). However, the use of this silane for this purpose relies on the esterification of the carboxylic groups of the peptides to form a covalent bond that immobilize them on the silanized surface. The ineffective esterification of the carboxylic groups entails polymerization of the peptide and possible racemization \(^{28}\).

Here, our objective was to modify titanium surfaces with a simple and reliable method to tether oligopeptides and thus form a stable bioactive coating. We present a 4 step process that covalently immobilizes TGF-β1 antagonistic peptides to titanium surfaces. Notably, we used malonic acid as crosslinker with the objective of obtaining a homogeneous peptide coverage of the surface by forming a selective covalent bond between the linker and primary amines of the peptide without polymerization and/or racemization. Our hypothesis is that a modified P17-peptide (MP17) tethered on titanium surfaces retains P17’s TGF-β1 inhibitory activity. The clinical goal is preventing peri-implant fibrosis, which eventually is beneficial for implant osseointegration.
EXPERIMENTAL SECTION

Materials

Bars of 10 mm in diameter of commercially pure titanium grade 2 (Ti) were purchased from Daido Steel Co. (Nagoya, Japan). 96% ethanol and acetone were purchased from Panreac S.A (Barcelona, Spain). Sodium hydroxide (NaOH), 2-propanol, anhydrous toluene, anhydrous cyclohexane, 3-aminopropyltrithoxysilane (APTES), malonic acid (Mal), dimethyl formamide (DMF), N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), N,N-Diisopropylethylamine (DIEA), Ethylenediaminetetraacetic acid (EDTA), glycine, methanol, sodium deoxycholate, Trizma base, Trizma hydrochloride, sodium dodecyl sulfate (SDS) and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent molecules, FITC cadaverine and 5-FAM-X,SE were purchased from Anaspec (Fremont, CA). Penicillin-streptomycin, L-glutamine, phosphate-buffered saline (PBS) and alpha minimum essential medium (α-MEM) were purchased from Gibco Life technologies (New York City, NY). Protease inhibitor cocktail, rabbit-anti-phospho-SMAD2 antibody and mouse-anti-rabbit peroxidase coupled secondary antibody were purchased from Cell Signaling (Danvers, MA). Chemiluminiscence detection kit Lumi-LightPLUS was purchased from Roche GmbH, (Mannheim, Germany). Acrylamide:Bis (29:1), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), bromophenol blue and dithiothreitol (DTT) were purchased form Bio-Rad (Hercules, CA). Human Recombinant TGF-β1 was purchased from Prospec (Ness-Ziona, Israel). MP17 peptide (H-KGGGGGKRIWFIPRSSWYERA-OH) is a peptide derivate from the P17 sequence with a lysine at the N-terminus aimed to directly react with the linker at the surface; followed by a chain of 5 glycines used to space the peptide from the surface and thus increase P17 activity. MP17 was synthesized by solid-phase peptide
synthesis method in an automatic APEX 396 multiple peptide synthesizer (Aapptec LLC and kindly furnished by Digna Biotech (Madrid, Spain).

Methods

Biofunctionalization process

The preparation of the functionalized surfaces was performed by means of a 4 step process. The surface was first activated with sodium hydroxide (Step 1), subsequently silanized using 3-aminopropytriethoxysilane (APTES) (Step 2) and then crosslinked with malonic acid (Step 3). Finally the immobilization of amino-containing fluorescent labels or MP17 oligopeptide was performed (Step 4).

Step 1 (NaOH (St. 1)): Ti discs were grinded with silicon carbide and mirror polished (Ra ≤ 50 nm) with subsequent alumina suspensions (1 µm and 0.05 µm mean size). After polishing, the samples were washed by subsequently 5 minutes of ultrasonication in ciclohexane, isopropanol, distilled water and acetone. The clean and polished titanium samples were immersed in NaOH 5 mol/l at 60 ºC for 24 h to form reactive –OH groups on the surface. After etching, the samples were immersed in distilled water during 30 minutes three times, washed with acetone and dried with N₂.

Step 2 (APTES (St. 2)): The surfaces were then immersed in a 30 mM APTES solution in anhydrous toluene at 70°C during 1h with agitation and nitrogen atmosphere. After silanization, the samples were sonicated in anhydrous toluene for 15 minutes to detach any loosen APTES molecules. Subsequently, the silanized samples were washed with ethanol, isopropanol, distilled water and acetone and dried with nitrogen.
Step 3 (Crosslink (St. 3)): Then, the silanized surfaces were treated by immersing them into a dimethylformamide (DMF) solution containing malonic acid (Mal) 10 mM, O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) 30 mM and diisopropylethylamine (DIEA) 100 mM. The crosslinker solution was used during 2 h at room temperature with agitation. Then the samples were washed in pure DMF twice.

Step 4 (MP17, St. 4): Covalent conjugation of oligopeptides on Ti surfaces was accomplished by immersing overnight the treated Ti discs into a DMF solution containing 0.2 mM of the MP17 peptide, or failing that, 0.2 mM of the fluorescent labels used for the fluorescence tests: the 5-FAM-X,SE or the FITC cadaverine. Ti discs were rinsed with deionized water and acetone.

Scanning electron Microscopy (SEM)

The surfaces were studied by means of a scanning electron microscope (SEM) (Zeiss Neon40, Zeiss, Germany) with an acceleration voltage of 8.0 keV and a vacuum chamber pressure of $10^{-9}$ mbar.

Water contact goniometry

Contact angle measurements were performed using the sessile drop method on a DM-CE1 goniometer (Kyowa, Japan). 3 µl drops of distilled water were dispensed on the sample surfaces. Water contact angles were measured using the FAMAS software (Kyowa, Japan) after 30 s of water contact; ie., when water contact angles were stable, .

Fluorescence Microscopy

Characterization by fluorescence analysis of the effectiveness of the chemical route used to functionalize the treated surfaces was performed. The samples were functionalized with 5-FAM-
X, SE or FITC cadaverine fluorescence molecules instead of using MP17 peptides. The use of these two different labels, which have different functional groups, allowed us to determine whether covalent reactions were taking place on the surface. The use of MP17 conjugated with a fluorescent label at the N-terminus was not possible since the fluorescent molecules compete with the surface for the available amine groups of the peptide; therefore, the covalent reaction between the peptide and the surface would be hindered.

A BX51 microscope (Olympus, USA) was used to obtain fluorescence pictures of the surfaces coated with label molecules. The microscope was equipped with a fluorescent lamp and an Olympus DP 25 (USA) digital camera. The exposure time for each taken picture was 2 s and all parameters were fixed to get comparable intensities. To obtain numeric values of fluorescence intensity the pictures were analyzed by means of Image J v1.45 software. Mean and standard deviation for fluorescence intensities of the different tested groups were analyzed.

X ray Photoelectron Spectroscopy (XPS)

XPS analysis was conducted with a Quantum 2000 spectrometer (Physical Instruments, USA) using a monochromated Al Kα beam of 1 mm spot size at 45° with respect to the analyzed surface and a step size of 1 eV (4 scans/sample). The high resolution analysis of the peaks of interest was performed at 0.1 eV step size (20 scans/sample). To detach any loosen molecules all samples were sonicated in distilled water for 2 hours prior the surface chemical analysis. Chemical composition and peak deconvolution were assessed using CASA XPS v. 2.3.16 software (Teignmouth, UK).
Mechanical and chemical stability of the coatings

All treated surfaces were mechanically challenged by ultrasonication in distilled water. Water contact angles and fluorescence visualization were performed after different times of ultrasonication. Chemical stability tests were performed immersing the different Ti samples in PBS at 37 ºC without sonication for different periods. The samples were analyzed by water contact goniometry before and after the thermochemical challenge.

Spectroscopic Ellipsometry

Ellipsometry is used to assess thickness of thin organic coatings when the roughness of the substrate is lower than the thickness of the investigated coating. For this reason, in this case we tested silicon wafers covered with a homogeneous titanium oxide layer that were purchased from the Nanotechnology Platform of the Parc Científic at the University of Barcelona (Barcelona, Spain). The coated wafers were activated by O₂ plasma treatment for 5 minutes, which did not modify the original topography and roughness of the wafer samples. Measurements were performed on surfaces after each step of the functionalization process and 2 hours of ultrasonication in water. The ellipsometry tests were performed using a VASE spectroscopic ellipsometer (J.A. Wollam Co., USA). Angles of incidence of the beam were 60° and 75° and spectra were collected for 500 nm-1100 nm wavelength rage and 15 nm pass. The data was analyzed using the VASE software from the ellipsometer manufacturing company. Silicon and TiO2 refractive indexes (n) and extinction coefficients (k) at the software’s database were used for modeling the organic coatings with a Cauchy dispersion model 31-33. Theoretical thicknesses of the layers were calculated by means of Chemdraw 3D Pro (Cambridgesoft, UK). The different
molecules where drawn in 3 dimensions, respecting the theoretical distances and angles of the different atomic bonds present in the molecules.

Western Blot analysis

MC3T3-E1 murine pre-osteoblast cells were cultured in serum free α-MEM. After 24 h, 0.7 ng/ml of TGF-β1 were dissolved in the same medium, exposed to the different Ti surfaces and incubated at 37°C for 30 minutes. Subsequently, the medium was transferred to the cells and incubated at 37°C for 1 hour. Then cells were lysed with RIPA buffer (50 mM Tris- HCl, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, plus protease inhibitors) and centrifuged (12,000 g, 10 min, 4°C). Total protein of supernatants was determined by the bicinchoninic acid assay (BCA Protein Assay; Pierce, Waltham, MA) and equal amounts were size-fractionized in SDS-PAGE under reducing conditions (100 V, 40 mA/gel), transferred to nitrocellulose membrane (60 V; 4°C, overnight) and stained with red-Ponceau. Membranes were blocked in TBS-T buffer (0.9% NaCl, 0.02 M Tris pH 7.5, 0.05% Tween-20, 5% BSA; 1 h; RT) previous to incubation with rabbit-anti-phospho SMAD2 antibody (1:500). Detection was performed with mouse-anti-rabbit peroxidase coupled secondary antibody in TBS-T buffer and an enhanced chemiluminescence (ECL) kit (Amersham, GE Healthcare, Waukesha, WI, USA). Bands were quantified with a digital image analyser (Quantity One Quantitation Software™; Bio-Rad, Hercules, CA, USA) on unsaturated x-ray films.

Statistical analysis

Statistically significant differences (p<0.05) among groups were assessed using non-parametric Kruskal-Wallis and multiple comparison post-hoc Mann-Whitney tests.
RESULTS AND DISCUSSION

Surface functionalization process

A 4-step functionalization process was developed to covalently immobilize oligopeptides to Ti surfaces. Initially, a surface activation was conducted by means of alkaline etching of the titanium surface with NaOH. This process provided the surface with high polarity and thus reactivity due to formation of abundant hydroxyl groups. This was because the combination of a) a highly intricate nanotopography that notably increased the real surface area of the substrate (Figure 1, upper row) with b) a modification of the chemical nature of the substrates from a mixture of titanium oxides (TiO$_2$, Ti$_2$O$_3$ and TiO) to a homogeneous sodium titanate (Na$_2$Ti$_3$O$_7$) layer, as previously demonstrated $^{34,35}$ and shown here by the detection of a strong Ti$^{4+}$ peak in the XPS spectrum (Fig. 1, lower row). The significant increase in hydrophilicity (significant decrease in water contact angle) of the surfaces treated with NaOH in comparison with the plain Ti surfaces (Figure 2) further confirmed the activation of the treated surfaces.

The second step of the functionalization process consisted of the silanization of the activated titanium surfaces using APTES. As previously established, hydrolysis of the ethoxy groups of the APTES molecules and subsequent reaction with the surface OH groups leads to a polycondensation reaction to form Ti-O-Si and Si-O-Si bonds $^{19}$. Silanization significantly decreased surface wettability of the activated surfaces (Fig. 2) due to the presence of hydrophobic alkyl chains in the APTES molecules. The drastic change in water contact angles before (Step 1) and after silanization (Step 2) suggested that a large amount of APTES molecules were retained on the treated surfaces.
In the third step of the functionalization process APTES silanized surfaces were innovatively treated with malonic acid as crosslinker and HBTU as coupling agent. Combined malonic acid and HBTU form an active diester that enables the nucleophilic-electrophilic reaction with the amino groups of APTES molecules. The later leads to the establishment of a stable amide bond between APTES and malonic acid molecules. This crosslinking process provides with a surface coated with active esters that can readily react with free amino groups of the oligopeptides, such as those provided by lysine residues in the peptide sequence as well as the peptide N-terminus. Thus, the fourth and final step was designed to enable specific immobilization of free-amine containing molecules to the titanium surface through the formation of a second amide bond between the malonic acid molecules and the oligopeptides. Since the peptide solution does not contain coupling agents polymerization of the peptide in solution is not feasible and use of strong bases is not needed, which avoids racemization\textsuperscript{36,37}. The effective formation of amide bonds in Steps 3 and 4 of the functionalization process is critical for attaining a successful immobilization of the crosslinking and bioactive molecules, respectively. To investigate the effective formation of amide bonds we anchored two different fluorescent molecules, 5-FAM-X,SE and FITC cadaverine, as functional surrogates for the malonic acid-HBTU and free-amine containing peptide molecules, respectively. These two fluorescein derivatives differ in the functional group, which is a primary amine in the case of FITC cadaverine and a succinimidyl ester in the case of 5-FAM-X,SE. Thus, 5-FAM-X,SE was anchored on the APTES-silanized surfaces and FITC cadaverine on surfaces with attached crosslinking molecules during Steps 3 and 4, respectively (Fig. 2 and 3). The resulting fluorescence intensities revealed that titanium surfaces retained notable amounts of the surrogate molecules during both Step 3 and 4 of the functionalization process. Higher intensities were assessed after Step 3 than after step 4, which can be attributed to
the additional strong electrostatic attraction between the APTES and 5-FAM-X,SE molecules \(^{38}\). These surfaces showed high and stable fluorescence intensities and correlated quasi constant water contact angles after sonication in water for periods longer than 3h (Figure 2). The later suggested that once all physisorbed molecules were released from the surface a significant amount of covalently bound molecules were successfully retained on the coating. Images obtained by fluorescence microscopy showed a homogeneous coating of the fluorescent molecules on the functionalized surfaces (Supplementary material).

Confirmation of the effective covalent immobilization of fluorescent molecules with primary amines through the aforementioned specific reactions is shown in Figure 3. The tested groups of treated surfaces with the designed potential to generate covalent bonds with fluorescent molecules; i.e, APTES with 5-FAM-X,SE (APTES(St. 2)-FAM) and malonic acid with FITC cadaverine (Crosslink(St. 3)-FITC), showed the highest stability of the fluorescence intensity after 20 h of sonication in water. However, the control groups with unspecific combinations of treated surfaces and fluorescent molecules did not retained a notable fluorescent signal after 20 hours of sonication, which suggested that most of the fluorescent molecules were just physisorbed by weak electrostatic and/or hydrophobic interactions with the substrate \(^{39,40}\).

XPS results further confirmed the validity and effectiveness of the functionalization process. Table 1 displays the molar percentage composition of the surfaces after each step of the functionalization process. On plain Ti surfaces, as expected, oxygen, titanium and carbon were the main elements detected. The presence of carbon reflects adventitious contamination from the ambient environment which mainly consists on oxidized aliphatic chains \(^{41}\). The high resolution
deconvolution of the Ti2p peak (Fig. 1) showed a small peak at low binding energy (453.3 eV), characteristic of metallic Ti. The most intense contribution was from Ti$^{4+}$ (854.5 eV), which is characteristic of the passive layer formed on Ti surfaces that is rich in TiO$_2$. Low signals for TiO (454.4 eV) and Ti$_2$O$_3$ (456.3 eV) were also detected. After surface activation by NaOH etching, the carbon C1s signal dramatically decreased, which suggested a thorough surface cleaning during alkaline etching. The Na1s, O1s signals and the Ti/O ratio increased according to the generation of a thicker sodium titanate layer $^{42}$. Silanization of the activated surfaces generated increased carbon C1s signal from the aliphatic chains of the APTES molecules and nitrogen N1s signal from the functional terminal group of the silanes. After immobilization of the crosslinking molecules further increase of the carbon C1s was detected, which revealed the presence of malonic acid molecules. Most interestingly, an increase in the nitrogen signal was also assessed. This suggested that the malonic acid molecules were esterified by combination with the HBTU coupling agent. The deconvolution of the high resolution N1s peaks (Fig. 4) for the surfaces after Step 2 revealed that about half of the amino groups of the anchored APTES molecules were in its protonated form. After Step 3, the deconvolution of the high resolution N1s peak (Figure 4) proved the formation of the amide bonds (N-C=O) between the silanes and the malonic acid crosslinking molecules. The presence of these chemical groups was also assessed by DRIFTS (Supplementary information).

Combined, all those results suggest that we successfully achieved the covalent immobilization of amino groups on titanium surfaces with high stability by a simple method that avoids peptide polymerization and racemization.

**MP17-peptide coatings**
We designed MP17 peptide, a modified P17 peptide to improve covalent bonding and retention of bioactivity of the TGF-β1 inhibitor peptide. The modification consisted of the addition of a lysine residue at the N terminus of the peptide followed by a chain of five glycines. The lysine residue contains a free amino group to react with the active esters of the functionalized surface through the APTES-Malonic acid route that has been previously described. The five glycines between the first lysine and the biologically active sequence was introduced to space the P17 sequence from the titanium surface as well as to allow mobility of the peptides as to improve the interfacial reactions with the biological entities; i.e., macromolecules and cells. Thus, retention of the biological efficiency of the peptide coatings was expected.

The build-up and properties of the peptide coatings were assessed by immobilizing the MP17 peptide on the functionalized titanium and analyzing the surfaces by means of contact angle goniometry, XPS, DRIFTS and spectroscopic ellipsometry.

After immobilizing the MP17 on the functionalized surfaces, XPS surveys revealed an increase of the N1s and C1s signals as well as the O/Ti ratio (Table 1). This results suggested that a considerable amount of peptide was deposited on the surface. Deconvolution of the nitrogen N1s high resolution peak (Fig. 4) showed only one peak that corresponded to the peptide bonds and covalent amide bonds (401 eV) and thus, the MP17 oligopeptide was covalent bonded to the surface. DRIFTS studies on the MP17 surfaces corroborated these results (Supplementary data).

The thermalchemical stability of the MP17 coatings was evaluated by contact angle goniometry (Fig. 5). The coated surfaces were immersed in water at 37 °C for 15 days and water contact angles on the challenged coatings were measured at different times. Even though we assessed a low-rate progressive reduction of the contact angle on all functionalized surfaces, after 15 days of incubation the surfaces were significantly less hydrophilic than after being activated by NaOH.
etching. This result indicates that the peptide coatings were highly stable with strong retention of the bioactive molecules after such period of immersion time. The slight reduction in water contact angle with immersion time was most likely related to hydrolysis of silanol groups of the APTES molecules \(^43\), which is the weakest link of the functionalization process.

The Cauchy models for the spectroscopic ellipsometry results revealed the successful buildup of the coatings during the functionalization process (Figure 6). The calculated thickness of the formed APTES layer after 2 h of sonication in water (0.52 nm) indicated that a strongly retained silane monolayer was obtained on the titanium surface \(^27,38,44,45\). After cross-linking with malonic acid+HBTU, the coating increased approximately 1 nm in thickness. This result correlates well with the theoretical atomic distances of the molecules present on the surface. After linking the MP17 peptide and 2 h of sonication in water, the thickness of the coating significantly increased due to the bonding and retention of the more bulky peptide molecules. The theoretical thickness of the MP17 coating was lower than the one calculated from the ellipsometry measurements and fitted to a Cauchy model. This result suggested the MP17 coatings were composed of a peptide multilayer, meaning that a physisorption process is always generated between the peptide and the surface, independently of whether a covalent reaction is taking place or not. The amount of peptide present on the surface was quantified to 36 nmol/cm\(^2\) (supplementary material) which suggests a multilayered peptide coating in correlation with the spectroscopic ellipsometry results.

**Bioactivity of MP17-peptide coatings**

High expression levels of phosphorilated SMAD2 (p-SMAD2) by cells when TGF-\(\beta\)1 is administered in MC3T3-E1 pre-osteoblast culture is the most reliable method to elucidate whether the TGF-\(\beta\)1 signaling pathway is activated. Figure 7 shows Western Blot results
demonstrating that the modified MP17 peptide was able to partially inhibit the activity of TGF-β1 when both administered free in solution and, most notably, immobilized on the titanium surface with the developed functionalization process. On titanium surfaces biofunctionalized with MP17 the expression of p-SMAD2 was significantly reduced in comparison with a non-biofunctionalized surface. This inhibition effect was even stronger on cells cultured on non-biofunctionalized titanium surfaces and exposed to the peptide in solution at high concentrations. Importantly, p-SMAD2 expression levels were very low but still detectable when exogenous TGF-β1 was not administered to the cells, which suggested an autocrine/paracrine activity of TGF-β1 on MC3T3-E1 pre-osteoblasts.

These results validate our hypothesis that the MP17-peptide tethered on titanium surfaces displays TGF-β1 inhibitory activity. Thus, these coatings represent a promising new approach for biofunctionalizing surfaces for inducing fast and reliable osseointegration that will be further in vitro and in vivo tested.

CONCLUSIONS

A new route to covalently immobilize oligopeptides on titanium surfaces has been developed. The functionalization of Ti with APTES and malonic acid led us to generate amide bonds between the free amino groups of the peptide and the treated surfaces. The surfaces were mechanically and chemically stable due to the covalent bonds formed between the organic molecules and the metallic surface. Also, MP17, a modified version of P17, a TGF-β1 inhibitor peptide, was immobilized on titanium surfaces using this functionalization process and retained the biological activity; i.e., the MP17-coatings reduced the effect of TGF-β1 on MC3T3-E1 cells.
This approach opens new perspectives in the field of biofunctionalization of surfaces for hard tissue repair and can be also used in applications to investigate and direct the stem cell fate.

ACKNOWLEDGMENTS

This work was partially supported by the University of Minnesota through a Grant-in-Aid of Research, Artistry, and Scholarly (CA), and the Spanish Ministry of Economy and Competitiveness (project MAT 2012-30706) (FJG). PS acknowledges financial support from the Spanish Minister of Economy and Competitiveness through a fellowship for research stays at international institutions (FPU-MEC). Parts of this work were carried out in the University of Minnesota I.T. Characterization Facility, which receives partial support from NSF through the MRSEC program. Parts of this work were carried out in the Institute for Bioengineering of Catalonia-IBEC.

References


Figure 1. Surface activation by alkaline etching. Upper row: SEM pictures of Ti surfaces before (left) and after (right), activation with NaOH etching. Lower row: deconvolution of the high-resolution XPS Ti2p peak of Ti surfaces before (left) and after (right) activation.
Figure 2. Effectiveness of the route of functionalization. Wettability (redish bars) and fluorescence intensity (greenish bars) of the treated Ti surfaces after different steps of the functionalization process. Results after different sonication periods are also shown. Plain Ti and NaOH treated samples were labeled with physisorbed FITC cadaverine molecules. APTES-silanized surfaces (Step 2) and silanized surfaces with anchored crosslinker (Malonic acid) (Step 3) were labeled with FAM-X(SE) and FITC cadaverine molecules, respectively to investigate the formation and stability of the covalent attachment of the fluorescent molecules.
Figure 3. Specificity and stability of the covalent reaction between treated titanium surfaces and fluorescent molecules. Percentage of retained fluorescence intensity of treated titanium surfaces and coated with 5-FAM-X, SE or FITC cadaverine molecules between different times (0h-2h, and 2h-20h) of ultrasonication in water at room temperature. * and # indicates groups differences with statistically significant differences (p<0.05).
Table 1. Chemical composition of treated surfaces. Quantification of chemical composition after each step of the functionalization process and final MP17 coating after ultrasonication for 2h in water.

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<th>C1s</th>
<th>Cl2p</th>
<th>N1s</th>
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<th>O1s</th>
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<th>Si2p</th>
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<td>54.5</td>
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<td>46.8</td>
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Figure 4: Analysis of amine and amide bonds after immobilization of molecules.
Deconvolution of high resolution N1s peaks obtained by XPS on treated titanium surfaces after immobilization of APTES (A: APTES (St.2)), immobilization of malonic acid (B: Crosslink (St.3)), and immobilization of MP17 peptides (C: MP17 (St.4)).
Figure 5. Thermochemical stability of the peptide coatings. Water contact angles of the treated titanium surfaces after different immersion times in water at 37 °C.
Figure 6. Thickness of coatings. Cauchy and theoretical thickness of the coatings after the different steps of the functionalization process from ellipsometry results. * indicates groups with no statistically significant differences (p≥0.05).
Figure 7. Bioactivity of MP17 peptides and coatings. Western Blot results for expression of p-SMAD2 on MC3T3-E1 preosteoblasts. TGF-β1 row: + TGF-β1 was administered to the cells at 0.7 ng/ml. - TGF-β1 was not administered (background control). MP17 row: + titanium surface was biofunctionalized with MP17-peptide. - titanium surface was not biofunctionalized. 200 µg/ml, titanium surface was not biofunctionalized but MP17 was administered to the cells in solution at a concentration of 200 µg/ml.